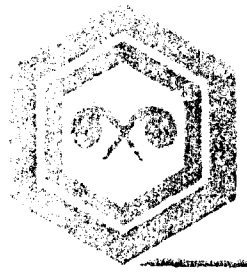

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BIODEGRADATION OF MUSTARD

Ronald J. Young

RESEARCH AND TECHNOLOGY DIRECTORATE

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Aberdeen Proving Ground, MD 21010-5423

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A literature search to identify microorganisms of potential value for the degradation of mustard was carried out. Selection of microorganisms was based on tolerance to low pH and chloride ions, conditions that retard mustard hydrolysis. Several bacteria able to degrade organic sulfides and/or sulfonium compounds under these conditions were identified. Fungi and yeasts are also of potential use, as are enzymes from halo- and thermophilic organisms. The major difficulty in the use of microorganisms and enzymes for mustard degradation is the low solubility of mustard in water.

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SUMMARY

The goal of biodegradation of mustard by direct microbiological action on the agent rather than on its hydrolysis product has been impeded by the chemical reactivity and insolubility of the agent. Microorganisms were inactivated by mustard which also was hydrolyzed during incubation in the aqueous medium used for growth of the microorganism. Microorganisms possessing an enzyme system functional against mustard or sulfonium compounds, intermediates in mustard hydrolysis, should be resistant to killing by the agent. With this assumption, a search of the literature was made to identify microorganisms able to degrade organic sulfur compounds and/or to grow under conditions, such as the presence of chloride ions, a relatively high concentration of mustard, and acid pH, in which mustard hydrolysis rate is low.

Some members of the genera Halomonas, Haloanaerobium, Halobacteroides and Sporohalobacter are involved in the degradation of organic sulfides. These microorganisms tolerate NaCl up to 1M and are found in ocean estuaries, along the sea shore, and in anoxic hypersaline waters and their sediments. Unidentified microbes present in anoxic salt marshes and one strain of Pseudomonas (MS strain) are able to degrade sulfonium compounds. Some strains of neutrophilic Thiobacillus and Pseudomonas, present in soil and aquatic regions rich in decaying organic matter, are also active in the degradation of organic sulfides. Although all the above microorganisms are neutrophiles, their ability to degrade organic sulfur compounds, particularly sulfonium compounds, and tolerance of chloride ions fulfills two of the criteria for use.

Less information is available on the degradation of organic sulfides by acidophiles. The acidophile Acidiphilium is currently under intensive study for use in the desulfurization of fossil fuels. Exploration of the application of this bacteria for mustard degradation is indicated. Acidophilic Thiobacillus appear to have limited use for mustard breakdown except for the halotolerant T. prosperus, originally isolated from a shallow geothermally heated seafloor. Acidic marine sites are attractive as sources of isolates for study.

Yeasts and fungi have not been used for the degradation of mustard, and there have been few reports on the utilization of organic sulfides by these microorganisms. This notwithstanding, these microorganisms are attractive candidates for use in degradation of mustard. Yeasts and fungi are acid tolerant and some are halotolerant. Fungi are rather universal in their utilization of organic matter. They are also valuable as a source of macro- and simple organic molecules such as exo- and endoenzymes, surfactants, and polymer, some of which may be

beneficial in mustard degradation.

The use of microbial enzymes rather than the microorganisms for mustard breakdown is a viable alternative. Enzymes of halophilic and thermophilic microorganisms are able to function in the presence organic solvents thereby alleviating the problem of mustard solubility in water. The ability of extracts or lysates of the thermophilic acidophile S. acidocaldarius to degrade mustard may be a good test of the enzymatic rather than the microbiological approach to mustard demilitarization.

The literature search has identified several microorganisms of potential use in the biodegradation of mustard. However, the critical component in biodegradation of mustard is the dispersion or solubilization of the agent in a medium in which the microorganism remain viable. Defining such a system is a necessary first step in any such endeavors.

PREFACE

The work described in this report was authorized under Project No. 10162622A553, CB Defense/General Investigation. This work was started in April 1993 and completed in October 1993.

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BIODEGRADATION OF MUSTARD

1. INTRODUCTION

Biodegradation of mustard stockpile is an attractive alternative to incineration or chemical treatment because of economic, safety and environmental concerns. Two studies have been carried out on the biological degradation of mustard. Bacteria isolated from soil previously exposed to mustard were either resistant or sensitive to mustard. The first isolates did not degrade mustard (1), while the second isolates, although sensitive to mustard itself, were able to utilize the hydrolysis product of mustard, thiodiglycol, as an energy and carbon source (2). This property of the second isolates, identified as Pseudomonas pickettii and Alcaligenes xylosoxidans ssp. xylosoxidans, coupled with prior alkaline hydrolysis of mustard, is the basis of a method with a high potential for the economical and safe degradation of stockpiles of mustard. There is nevertheless a place for a biological procedure that degrades mustard directly, and completely to environmentally benign compounds. For example, the final products from biological degradation could be sulfate, chloride and carbon dioxide. The reconciliation of the intractable chemical, physical, and toxic properties of mustard with conditions suitable for growth of microorganisms presents a formidable, but perhaps not an insurmountable, obstacle to development of biological methods for its destruction. The lack of success thus far in attempts to degrade mustard directly suggests that exploration of alternate approaches would be appropriate. Thus microorganisms other than neutrophilic bacteria, and from habitats other than mustard treated soils are worthy of consideration. In this report, the properties of mustard pertinent to the use of microorganisms are considered, the result of a survey microorganism of potential use for the biological degradation of mustard is presented, and possible methods for the biological degradation of mustard are discussed.

2. PROPERTIES OF MUSTARD IN RELATION TO MICROORGANISMS

a. Chemical

The crux of any procedure for the direct biological degradation of mustard (I) is the stability and solubility of mustard in water, as growth of microorganisms occurs in aqueous medium. Hydrolysis of mustard is complex (3,4). The rate determining step is the formation of a cyclic sulfonium ion (II) which rapidly reacts further with water to produce thiodiglycol (IV)

as the final product (Figure 1). The end product can react with the cyclic sulfonium intermediates (I, II) to produce branched sulfonium ion intermediates (V-VII). Chloride ion inhibits the formation of the cyclic sulfonium ions, and low pH results in reversal of hydrolysis. Further, hydrolysis of the branched sulfonium intermediates is slow, is dependent on the hydroxide ion concentration, and is negatively correlated with the concentration of the reactant, the sulfonium intermediates (VI,VII) (4). Mustard is poorly soluble in water, and concentrations sufficient to retard hydrolysis require use of agents able to increase its miscibility with water. In this respect an apolar organic solvent or detergent would be desirable as mustard hydrolysis rate is significantly reduced in such solvents or detergent. These considerations point to a desirability for salt tolerant, acidophilic microorganisms that are resistant to organic solvents, neutral surfactants, or surfactants from biological sources.

b. Biological

An important consideration in using microorganisms to degrade mustard is the cytotoxic properties of the chemical. The primary target of mustard in the microbial cell is DNA resulting in inhibition of replication. Other cellular processes are apparently unaffected as cells continue to grow, with unbalanced growth as the cause of cell death (5). This seeming insensitivity of proteins to mustard maybe due to a low intracellular concentration of mustard, commensurate with the low concentration of mustard required to induce cell death, and the poor solubility of mustard in water. Microorganisms possessing an enzyme system capable of degrading the agent may be able to continue replication when exposed to low concentrations of mustard. The consequences of exposure of microorganisms to the higher concentrations of mustard that is desirable for the retardation of its hydrolysis is unclear. The intracellular mustard concentration would most likely also be high, but the species of mustard predominately present intracellularly, their stability, and reactivity in a heterogeneous milieu maybe quite different from what has been observed in a homogeneous solution. A careful balance of the conditions to maintain stability of mustard and the viability, even of salt and acid tolerant microorganisms, is necessary.

3. MICROORGANISMS

a. Halophilic Microorganisms

The rate of hydrolysis of mustard in water is retarded six fold in the presence of 0.14M NaCl (6). Higher concentrations of NaCl

Mustard Hydrolysis

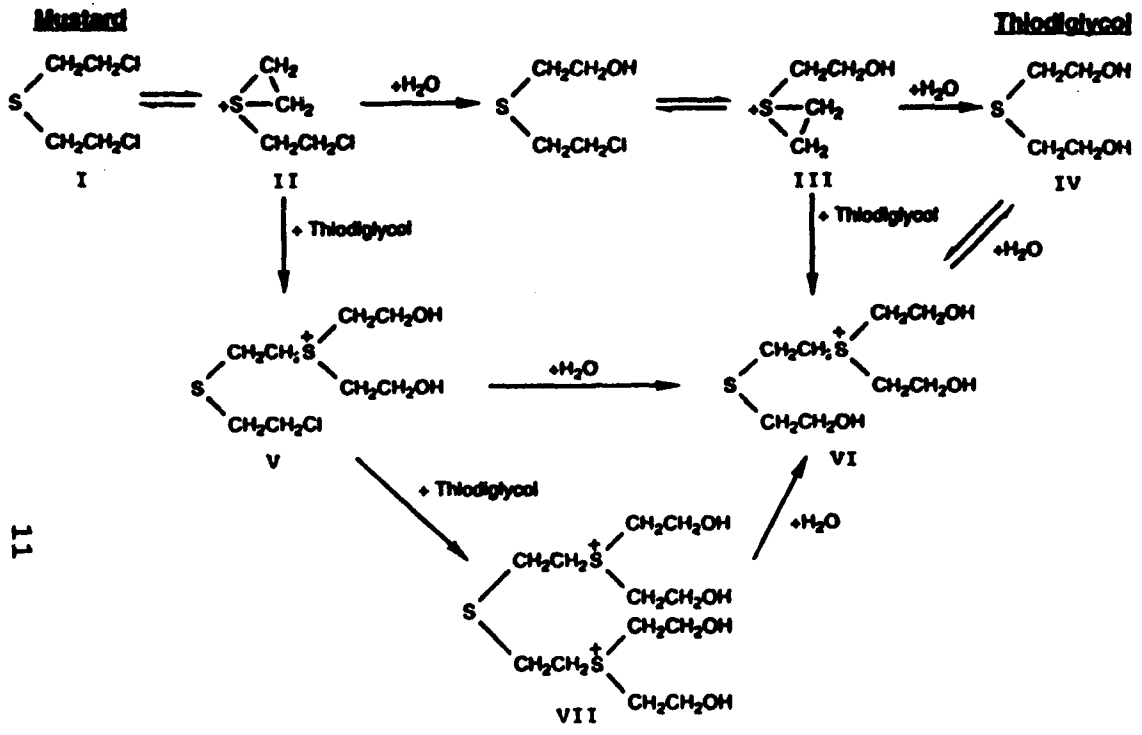

Figure 1. Hydrolysis of mustard. The rate determining step in the hydrolysis of mustard (I) is the formation of the cyclic sulfonium ion (II). Thiodigylcol (IV) is rapidly formed and this in turn reacts with the cyclic sulfonium intermediates to give the sulfonium intermediates (V-VII) whose hydrolysis is pH dependent. Chloride ion retards the formation of the cyclic sulfonium ions.
Adapted from reference 2

may retard the rate of hydrolysis further. The beneficial concentration of NaCl is limited, not by the tolerance of microorganisms for salt, but by the extraordinary low solubility of mustard in salt solutions as extremely halophilic bacteria are able to grow in saturated salt solution. The need to maximize the concentration of mustard in order to enhance stability will undoubtedly limit the salt concentration to much less than 1.5 M, conditions suitable for the slightly tolerant category of halophilic bacteria (7). This ceiling on NaCl concentration effectively eliminates consideration of members of the aerobic halophilic archaebacteria family, the family Halobacteriaceae, as the lower range of salt concentration for growth for members of this family is 1-1.5 M (8,9). Halotolerant bacteria of potential interest include those belonging to the genus Halomonas and the genera Haloanaerobium, Halobacteroides and Sporohalobacter as they can be cultured in lower salt concentrations. Members of the genus Halomonas eg H. elongata and H. halodurans show great flexibility for salt and are able to grow in salt concentrations between 0.016 M to 5.5 M (10), whereas those of Haloanaerobium, Halobacteroides and Sporohalobacter require a higher range of salt concentration, 0.45 M to >1.0 M (11). Halomonas species have been isolated from a solar saltern, an estuary and from the Pacific ocean. The Haloanaerobium, Halobacteroides and Sporohalobacter species are obligate anaerobes whose habitats are anoxic hypersaline waters, their sediments, and solar salterns. All microorganism species mentioned above are chemoorganotrophs and have possible applications in the removal of organic waste (10,11). Haloanaerobium praevalens is of particular interest in the present context as the organism degrades methionine producing methylmercaptan as a product (12). Halobacteroides strains have been shown to use methanethiol as the sole sulfur source for growth (13).

Anaerobes present in anoxic hypersaline environments, anoxic salt marsh and marine-ocean sediments are involved in metabolism of sulfur compounds and sulfur cycling (13,14), and samples from such sources may produce useful isolates. For example ethylated sulfur compounds are converted to ethane by methanogenic bacteria (15a). Dimethylsulfoniopropionate $(CH_3)_2\text{-}S^+\text{-}(CH_2)_2\text{-}COOH$, found in some algae, is degraded by microorganisms in anoxic salt marsh sediments to dimethylsulfide and acrylic acid, or 3-mercaptopropionic acid and methanethiol (15,16). Dimethylsulfide and methanthiol are in turn converted to H_2S and methane by sediment microorganisms such as sulfate reducers (13,14,16-18). By analogy, the cyclic sulfonium ion derived from mustard might be degraded to less toxic compounds such as ethylene sulfide, ethylene chloride, ethylene and 2-chloromercaptoethanol, and the sulfonium ion aggregates to a number of compounds including vinyl alcohol, and ethanol among others.

The end products of degradation of an organic sulfur compound by

an anaerobic microorganism are H_2S and methane or a sulfide, and an aerobic microorganism would be necessary to oxidize the sulfide to the more environmentally benign sulfate. Although the goal of degrading mustard to environmentally benign products may not be realized by anaerobic halophilic microorganisms, they nevertheless may, at least, serve in a first detoxifying step, since some species are able to cleave a carbon-sulfur bond, or perhaps more correctly, a methyl-sulfur bond. Mixed cultures may be necessary to achieve the desired degradation to H_2S and methane. As an aside, the enzyme involved in the degradation of dimethylsulfoniopropionate (19) deserves consideration as a device for protection against mustard exposure.

Microorganisms present in hypersaline, marine or aquatic environments are, with few exceptions neutrophilic (20a). The requirement for neutrality is not incompatible with mustard stability as the multimeric sulfonium chloride intermediates in mustard hydrolysis have some degree of stability in neutral conditions particularly in the presence of chloride anions (4). The abnormally high intracellular salt concentration of some eubacteria such as the Haloanaerobium, Halobacteroides and Sporohalobacter species (10) may also contribute to intracellular stability and afford a measure of protection to the toxic action of mustard.

Although many microorganisms tolerant of salt are known, it is unlikely that mustard would be soluble in solvent mixtures at the sodium chloride concentrations discussed above. Mustard hydrolysis is retarded by the chloride anion and the retardation is enhanced under apolar conditions. Thus the chloride concentration need not be high to stabilize mustard under these conditions. Quaternary ammonium chlorides are a source of chloride anions, and they may be an alternative, or even preferred over a very low concentration of sodium chloride because of their higher solubility in organic solvents.

b. Colorless Sulfur Bacteria

Bacteria utilizing reduced sulfur compounds as an energy source for growth are collectively known as colorless sulfur bacteria. The sulfur compounds are oxidized to sulfate either aerobically or anaerobically in the presence of nitrate. Except for the genera Thiobacillus, Sulfolobus and Acidianus, colorless sulfur bacteria are neutrophilic (20). The requirement for neutrality need not be an impediment to the use of colorless sulfur bacteria as hydrolysis of mustard even at neutral pH and in the absence of salt is significantly retarded if the concentration of mustard is sufficiently high. An acid (HCl) environment is preferred as this favors the reversal of both hydrolysis and the formation of the cyclic sulfonium ion. These reactions, however,

require strongly acidic conditions.

b.1. Neutrophilic Sulfur bacteria

This group of bacteria encompasses 18 genera whose members display a wide diversity of nutritional requirements and physiological types (20). Their habitats include aquatic regions, sediments, ocean hydrothermal vents, waste water treatment plants, soils, and any environment where reduced sulfur compounds are present. Studies of the use of organic sulfides rather than inorganic sulfides, hydrogen sulfide, or salts such as thiosulfate, as substrates by this group of bacteria appear to be restricted to the genera Hyphomicrobium, Thiobacillus and Pseudomonas. The naturally occurring methyl sulfide is oxidized to sulfate by Hyphomicrobium S sp., Hyphomicrobium EG (21-23), Thiobacillus thioparus TK-m and Thiobacillus MS1 (24,25), but only to dimethyl sulfoxide by Pseudomonas acidovorons DMR-11 (26). Methyl sulfide is also degraded by an obligately chemolithoautotrophic, bacillus T5, isolated from a microbial mat in the North Sea (27), an unidentified bacterium present in surface seawater (28), and facultative chemolithoautotrophs, tentatively identified as Thiobacillus strains E3-E7 isolated from garden compost, cattle manure, marine mud, pond water and moss, respectively (29). Thiobacillus thioparus is an obligate autotroph, and other Thiobacillus autotrophs as well as Thiobacillus facultative heterotrophs (29a) may also be able to use organic sulfur compounds as substrates. Only the Thiobacillus and, perhaps, Pseudomonas strains appear to have potential interest for the degradation of mustard as Hyphomicrobium is an obligate methylotroph. Nevertheless, a Hyphomicrobium species or other unidentified microorganisms present in soil samples were reported to oxidize methyl and higher sulfides to sulfate (30, 31).

Methyl and higher sulfides are the end products of the action of a variety of aquatic and terrestrial microorganisms on sulfur amino acids, and other sulfur containing compounds from decaying organic matter (32,33). The sulfur amino acids are themselves sulfides and these microorganisms may be able to utilize mustard for growth. Thus sediment, soils with decaying organic matter, and aquatic environments appear to hold promise as sources of microorganisms for which mustard maybe a substrate. It should be noted, however, that at neutral pH and in the absence of salt, the predominate chemical species would not be mustard itself, but the sulfonium aggregates. For this reason a strain of Pseudomonas (MS strain) which is able to use trimethylsulfonium chloride as sole carbon source (34-36), deserves consideration for study in the biological degradation of mustard even though the substrate is a C_1-sulfur compound, and dimethylsulfoniopropionate, discussed above, cannot replace trimethylsulfonium chloride as a substrate. Bacterium 5H2 also can grow on trimethylammonfum chloride, and since it is a

facultative methylotroph (37), it is a more attractive candidate.

Mustard sulfoxide is much less toxic than mustard as it does not react with proteins under normal physiological conditions (5). Bacteria oxidizing sulfides to the sulfoxide should receive more than a passing thought with respect to their action on mustard.

b.2. Acidophilic Sulfur Bacteria

Two of the three genera of colorless sulfur bacteria, Sulfolobus and Acidianus are thermophilic and are not considered further. Members of the third, Thiobacillus, are found in soil and water around mineral and coal mines, leaching dumps, acid soils and acid lakes such as volcanic lakes. The aerobic chemolithotropic Acidobacillus converts inorganic sulfides into sulfuric acid. Consequently studies have centered on their corrosive properties and use in mineral leaching rather than on their ability to use organic sulfides as substrates. Some thiobacillus are capable of growing as heterotrophs in the presence of low concentrations of substrate e.g. Thiobacillus rubellus, Thiobacillus delicatus (38), Thiobacillus acidophilus (39), and Thiobacillus cuptinus (40).

c. Other Acidophilic Microorganisms

A new genus of acidophiles, Acidiphilium, has recently been described (41). Members of this genus, originally isolated as contaminants of Thiobacillus species and present in coal and mineral sulfide environments, are mesophilic, acidophilic and heterotrophic. Many species of this genus have been described (39,42-46). The Acidiphilium strains vary a great deal in their ability to utilize organic compounds for growth, but all are heterotrophs, unable to use sulfur or inorganic sulfides for growth. The Thiobacillus and Acidiphilium species appear to be attractive candidates for further study. Possible uses for the latter species are under consideration (46a).

d. Considerations of Acidophily

Acidophiles are capable of growth at a pH of 1 (range 1-4). The cytoplasmic pH is, however, close to neutral, 5.5-6.7 when the external pH is 2-4 (47). In contrast, the pH of the periplasm of acidophilic gram-negative bacteria is thought to be close to the pH of the growth medium (48). The chemiosmotic consequences of this pH gradient is a tendency for exclusion of permeant cations, but not of uncharged chemical species or anions, due to the existence of a positive membrane potential in actively respiring bacteria (47). This means that the sulfonium species are denied entry into the acidophile, but not the uncharged

mustard sulfide. The SO_4^{2-} anion, resulting from oxidation of sulfide, is non permeant and does not react with mustard. The bacterial membrane is permeable to Cl^- (47,49), the anion of importance with respect to mustard stability, and maintenance of the uncharged mustard. Even though membrane polarity is reversed when the external pH is lower than about 2.5 (50), Cl^- anions are still able to enter because of the chloride porter system (48). Thus, while it is important for Cl^- anions to be present, the concentration should not be high, <3% (45,51), to avoid inhibition of growth due to a high internal concentration of this anion. Thiobacillus prosperus, isolated from a shallow geothermally heated seafloor, is a halotolerant (6% NaCl) acidophile. Although this organism is an obligate chemolithotroph (20a) other obligate chemolithotrophs have been shown to be able to degrade simple organic sulfides (27). Acidic marine areas appear attractive as potential sites for investigation as sources of isolates. Another possible source of Cl^- tolerant acidophiles is the stomach as the acidity in this organ is results from HCl. Ruman microorganisms have been shown to degrade organic sulfur compounds including sulfonium compounds (52).

4. COAL AND OIL

Combustion of fossil fuels generates oxides of sulfur resulting in the major environmental problem of acid rain. Microbial removal of sulfur from the fuels before combustion has received a great deal of attention as a means for alleviating this problem (53-59). Aromatic sulfides are the major sources of organic sulfur in the fuels. Coal in addition contains iron sulfide, pyrites, and sulfate. The inorganic sulfide is readily removed by the action of Thiobacillus ferrooxidans, Thiobacillus thiooxidans and Sulfolobus acidocaldarius, but the goal of organic sulfur removal from fossil fuels is still being pursued. Although the structure of the organic sulfides in fossil fuels is quite different from mustard or of the related sulfonium ions, it is instructive to consider the studies with the fossil fuels since the methods used and results obtained may be applicable to the degradation of mustard.

a. Coal

Sulfur in coal is covalently linked to carbon and is an integral component of the structure of coal. Thus it has proved difficult to desulfurize coal by microbial means. Dibenzothiophene (DBT) is universally used as a model compound in studies seeking microorganisms for the removal of sulfur from coal. Many reports of the bacterial degradation of DBT in the presence of a carbon source are available, but there are fewer reports of bacteria using DBT as source of carbon and sulfur. Bacteria cometabolizing DBT include members of the genera Pseudomonas (60-65), Beijerinckia (66), Rhodococcus, and Bacillus (67,68),

Corynevbacterium sp. (69), and unidentified microorganisms (70,71), including yeasts (72). Rhizobium sp., Acinetobacter sp. (59), Brevibacterium sp. (73), Sulfolobus acidocaldarius (74), and an unidentified microorganism isolated from a deep sea thermal vent (75) are able to use DBT or other sulfides as a source of carbon and sulfur.

Two pathways, neither cleaving the carbon-sulfur bond, have been identified for the degradation of DBT: oxidation of the sulfur to the sulfoxide and sulfone, and cleavage of one of the benzene rings (76). All microorganisms studied to date, except for Sulfolobus acidocaldarius, Brevibacterium sp., Corynevacterium sp., and perhaps several unidentified microorganisms (72,77), degrade DBT by either of the two pathways. The exceptions cleave the carbon-sulfur bond of DBT via the sulfone forming sulfate (69,72,73,77), or incorporate the sulfur into the biomass (68,78). Sources for the isolates were garden soil, environments surrounding petroleum refineries, coal storage areas and strip mines, and oil and coal tar contaminated soil. Enrichment culture techniques, a sulfur bioavailability assay (55), and chemical mutagenesis (55,60) were used for the isolation of the microorganisms.

Although many microorganisms are able to degrade DBT, a suitable process for the microbial desulfurization of coal is not at hand (53-56). One problem is the dissimilarity of the growth conditions of bacteria used for desulfurization: microorganisms for removal of organic sulfur are neutrophilic and those required for inorganic sulfur removal are acidophilic. As a possible solution, attention has recently been focused on the acidophilic heterotroph Acidiphilium, as this bacterium coexists with the acidophilic Thiobacillus used in removal of inorganic sulfur. An interesting approach has been the application of techniques of molecular genetics with the aim of introducing characteristics to enhance desulfurization, such as the ability to degrade organic sulfur compounds, into Acidiphilium (79,80).

b. Oil

DBT and representative organic sulfides present in oil are degraded by species of Pseudomonas, a Flavobacterium sp., a Xanthomonas sp. (81-83), and fungal cultures (84) without releasing sulfur as sulfate. The carbon-sulfur bond of DBT and model compounds are cleaved anaerobically by species of Desulfovibro (85,86). Successful desulfurization of oil has been demonstrated with Desulfovibro (87) and unidentified microorganisms in marine water samples (88). One major obstacle in oil desulfurization is the immiscibility of oil with water. Studies with DBT suggest that degradation would be improved by increasing miscibility with a solvent (81,89). Interesting developments in this area of miscibility is the claim for

desulfurisation of oil by a membrane extract of Bacillus sphaericus and Rhodococcus rhodochrous contained in a organic medium (90), and the use of microemulsions of crude oil and the microorganism or extracts of the microorganism (91).

c. Application to Mustard Degradation

The studies with oil and coal amply demonstrate the existence of many bacteria with the ability to metabolize or cometabolize organic sulfides. All such bacteria, except for Acidiphilium, are neutrophiles and thus do not possess any advantage over those isolated by Harvey and DeFrank (2) for the degradation of mustard. The acidophile Acidiphilium is deserving of study for mustard degradation. The techniques of selective enrichment in sulfur deficient medium, chemical mutagenesis, and molecular biology for the introduction of characteristics for desulfurization eg. the plasmid-encoded DBT degradation gene (92) into a suitable recipient (acidophiles) are methods with possible application for mustard degradation. Biosurfactants may also be valuable as a solution to problems of mustard-water miscibility.
Bacterial extracts in organic solvents, or microemulsions of mustard and the extracts are other possible approaches. Stabilization of mustard by chloride anions should remain in the forefront of any scheme.

5. YEAST AND FUNGUS

Yeast and fungus have received relatively little attention as microorganisms for the degradation of sulfur compounds. Many studies have made passing mention of the presence of yeast and fungus in isolates of bacteria which degraded sulfur compounds, but it is not clear in these studies if the yeast or fungus possessed the ability to degrade sulfur compounds. Only in isolated instances have the identity of the yeast or fungus been established, presumably because in most studies they constituted a minority of the microorganisms in the isolate. Two yeast and 9 fungal isolates were obtained from oil contaminated soils in the Ogoni area and Ibadan, Nigeria. All were able to grow on petroleum hydrocarbons. The yeasts were not identified and 4 of the 9 fungal isolate were identified as species of Aspergillus, Cochliobolus, Monilia, and Trichoderma (93). Oil was degraded by fungal isolates from soil and the sea from different areas of Kuwait. The isolates were identified as Aspergillus terreus, A. sulphures, Mucor globosus, Fusarium sp. and Penicillium citrinum (94). Aureobasidium pullulans, commonly known as black yeast, has been used to clean oil spills in the ocean and inland waters (95). Although yeast and fungus have been successfully used in the degradation of oil, and solubilization of coal (96-101), it is not clear if the sulfur compounds present in these fuels are broken down and the fate of organic sulfur is unknown.

other fungal species, Mortierella isabellina NRRL 1757, Cunninghamella elegans, and Rhizopus arrhizus are able to oxidize the sulfur of aromatic and heterocyclic sulfides, including the model compound DBT, to the sulfoxide and sulfone (102-104), while dimethyl sulfide was oxidized to the oxide by an unidentified fungus (105). Ligninase, an extracellular enzyme produced by the ligninolytic fungus, Phanerochaete chrysosporium, catalyzed the oxidation of thianthrene, a heterocyclic sulfide present in coal (106). This fungus is able to solubilize coal (107) and it is possible that other oil and coal degrading fungi such as those discussed above may also possesses sulfur oxidation potential. One such fungus, Paecilomyces sp. TLi, solubilized coal under acid conditions, cleaved the carbon-sulfur bonds of DBT degrading it to 2,2'-dihydroxybiphenyl, and broke down dibenzylsulfide to a variety of products (108).

The above studies suggest that yeasts and fungi have limited application as microorganisms for the demilitarization of mustard. However, only a few species have been tested for their ability to degrade organic sulfur compounds, and the studies have concentrated on aromatic and heterocyclic sulfides which are recalcitrant to microbial breakdown. The fungi have several attractive attributes that warrant consideration for their use in the degradation of mustard. First, yeasts and fungi are generally acid tolerant and some are halotolerant (109,110). Second, fungi are versatile in substrate utilization, able to metabolize a wide spectrum of foreign substances including chlorinated compounds, polyaromatic hydrocarbons, pesticides and dyes, and have been extensively used in treatment of industrial waste, in waste water treatment, in the degradation of xenobiotics, and in biotechnology (111-114). Third, fungi are the source of a diverse group of substances ranging from macromolecules to simple organic compounds that have industrial use (115). These substances include enzymes and lipids (biosurfactants) some of which are produced extracellularly (116,117), and are of potential use in the breakdown of mustard such as the extracellular enzyme of Phanerochaete chrysosporium (106,118).

6. BIOCATALYTIC APPROACHES

Halophilic and thermophilic microorganisms have not been considered thus far because conditions required for growth present problems such as mustard-water miscibility in the high salt concentrations, and mustard stability in high temperatures, respectively. The enzymes produced by these microorganisms have interesting and unusual properties that encourage a biocatalytic approach to mustard degradation. The intracellular concentrations of salt (KCL and NaCL) in halophilic bacteria are

high and their intra- and extracellular enzymes are able to function under conditions of low water potential. Thus the enzymes are active in aqueous solutions high in salt or organic solvents, and are stable at room temperature (119-123). Enzymes from thermophilic bacteria and fungi are thermostable, show resistance to denaturation by detergents, chaotropes and organic solvents, to proteolytic cleavage, to chemical reactions that result in denaturation, and perhaps, to pH extremes (122-127). These properties suggest that it would be feasible to breakdown mustard enzymatically in non-aqueous media (128,129), or a homogeneous aqueous-organic medium under conditions of pH, salt and mustard concentrations that are consistent with resistance of mustard to hydrolysis. This possibility is open to test with the thermophilic acidophiles S. acidocaldarius and S. solfataricus. These two bacteria degrade sulfides present in coal (130-132), but are unsuitable as organisms for mustard degradation because of the high temperature required for growth. Extracts or lysates of the two bacteria may show activity against mustard when incubated with the agent under suitable conditions in a organic-aqueous medium. Microorganisms whose habitats are rich in reduced sulfur compounds such as thermal sulfur springs, soil in volcanic areas, warm environment surrounding coal mines and coal storage areas, thermal vents in oceans, sludge and waste treatment plants may possess enzyme systems able to degrade organic sulfides (127,133). Screening of extracts or lysates of these microorganisms for mustard degradation activity may be an avenue for exploration.

7. CONCLUSION

The foregoing discussion has identified microorganisms with potential use in the biodegradation of mustard. Success in the biodegradtion of mustard hinges not on tolerance of the microorganism for conditions that enhances mustard stability, but on mustard solubility, and viability of the microorganism in systems that are able to disperse or solubilize mustard. Such systems, whether detergent or organic solvent-based, should be defined and evaluated as a first step. Fungi should receive serious consideration as they often show more resistance to solvent insult than bacteria, and some are producers of detergents and exoenzymes that may be useful in mustard degradation. Enzyme degradation of mustard may eliminate solubility and viability problems.

LITERATURE CITED

(1) Dennis, J. and White, WE. Deoxyribonucleic acid (DNA) repair in mustard-resistant bacteria. CRDEC-TR-87043.

(2) Harvey, SP. and DeFrank, JJ. Biodegradation of chemical warfare agents:Demilitarization applications. Proc. Army Sci. Confer., June 1992, Orlando FA.

(3) Yang,Y-C., Baker, JA. and Ward, RJ. Decontamination of chemical warfare agents. Chem. Rev. 92:1729-1743 (1992).

(4) Yang, Y-C., Szafraniec, LL., Beaudry, WT., and Ward, RJ. Kinetics and mechanism of the hydrolysis of 2-chloroethyl sulfides. J. Org. Chem. 53:3293-3297 (1988).

(5) Papirmeister, B.,Feister AJ., Robinson SI., and Ford, RD. Medical defense against mustard gas: Toxic mechanisms and pharmacological implications. CRC Press, Boca Raton, Florida, 1991.

(6) Bartlett, PD. and Swain, CG. Kinetics of hydrolysis and displacement reactions of β-chloro-β'-hydroxydiethyl sulfide (mustard chlorohydrin). J. Amer. Chem. Soc. 71:1406-1415 (1949).

(7) Larsen, H. Halophilic and halotolerant microorganisms-an overview and historical perspective. FEMS Micro. Rev. 39:3-7 (1986).

(8) Tindall, BJ. and TrÜper, HG. Ecophysiology of the aerobic halophilic archaebacteria. System. Appl. Microbiol. 7:202-212 (1986).

(9) Tindall, BJ. The Family Halobacteriaceae in The Prokaryotes, Ed. A. Balows, HG. TrÜper, M. Dworkin, W. Harder and K-H. Schleifer. Chap. 34, second edition, Springler-Verlag, N.Y. 1992.

(10) Vreeland, RH. The Family Halomonadaceae in The Prokaryotes, Ed. A. Balows, HG. TrÜper, M. Dworkin, W. Harder and K-H. Schleifer. Chap. 167, second edition, Springler-Verlag, N.Y. 1992.

(11) Oren, A. the Genera Haloanaerobium, Halobacteroides, and Sporohalobacter in The Prokaryotes, Ed. A. Balows, HG. TrÜper, M. Dworkin, W. Harder and K-H. Schleifer. Chap. 84, second edition, Springler-Verlag, N.Y. 1992.

(12) Zeikus, JG., Hegge, PW, Thompson, TE. Phelps, TJ., and Langworthy, TA. Isolation and description of Haloanaerobium praevalens gen. nov., and sp. nov.,an obligately anaerobic halophile common to Great Salt Lake sediments. Curr. Microbiol. 9:225-233 (1983).

(13) Kevbrin VV. and Zavarzin, GA. Methanethiol utilization and sulfur reduction by anaerobic halophilic saccharolytic bacteria. Curr. Microbiol. 24:247-250 (1992).

(14) Oren A. Anaerobic degradation of organic compounds at high salt concentrations. Antonie van Leeuwenhoek 54:267-277 (1988).

(15a) Oremland, RS., Whiticar, MJ., Strohmaier, FE., and Kiene, RP. Bacterial ethane formation from reduced, ethylated sulfur compounds in anoxic sediments. Geochim. Cosmochim. Acta 52:1895-1904 (1988).
(15) Kiene, RP. and Visscher, PT. Production and fate of methylated sulfur compounds from methionine and dimethylsulfoniopropionate in anoxic salt marsh sediments. Appl. Environ. Microbiol. 53:2426-2434 (1987).
(16) Kiene, RP. and Taylor, BF. Demethylation of dimethylsulfoniopropionate and production of thiols in anoxic marine sediments. Appl. environ. Microbiol. 54:2208-2212 (1988).
(17) Kiene, RP., Oremland, RS., Catena, A., Miller, LG. and Capone DG. Metabolism of reduced methylated sulfur compounds in anaerobic sediments and by a pure culture of an estuarine methanogen. Appl. Environ. Microbiol. 52:1037-1045 (1986).
(18) Kiene, RP. Dimethylsulfide metabolism in salt marsh sediments. FEMS MIcrobiol. Rev. 53:71-78 (1988).
(19) Cantoni, GL. and Anderson, DG. Enzymatic cleavage of dimethylpropiothetin by Polysiphonia lanosa. J. Biol. Chem. 222:171-177 (1956).
(20a) Huber, H. and Stetter, KO. Thiobacillus prosperus sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field. Arch. Microbiol. 151:479-485 (1989).
(20) Robertson LA. and Kuenen JG. The Colorless Sulfur Bacteria in The Prokaryotes, Ed. A. Balows, HG. TrÜper, M. Dworkin, W. Harder and K-H. Schleifer. Chap. 16, second edition, Springler-Verlag, N.Y. 1992.
(21) De Bont, JAM., Van Dijken, JP. and Harder W. Dimethyl sulphoxide and dimethyl sulphide as a carbon, sulphur and energy source for growth of Hyphomicrobium S. J. Gen. Microbiol. 127:314-323 (1981).
(22) Suylen, GMH., Stefess, GC. and Kuenen, JG. Chemolithotrophic potential of a Hyphomicrobium species, capable of growth on methylated sulphur compounds. Arch. Microbiol. 146:192-198 (1986).
(23) Suylen, GMH. and Kuenen, JG. Chemostat enrichment and isolation of Hyphomicrobium EG, a dimethyl-sulphide oxidizing methylotroph and reevaluation of Thiobacillus MS1. Antonie van Leeuwenhoek 52:281-293 (1986).
(24) Kanagawa, T. and Kelly, DP. Breakdown of dimethyl sulphide by mixed cultures and by Thiobacillus thioparus. FEMS Microbiol. Lett. 34:13-19 (1986).
(25) Kanagawa, T. and Mikami E. Removal of methanethiol, dimethyl sulfide, and hydrogen sulfide from contaminated air by Thiobacillus thioparus TK-m. Appl. Environ. Microbiol. 55:555-558 (1989).
(26) Zhang, L., Kuniyoshi, I., Hirai, M. and Shoda, M. Oxidation of dimethyl sulfide by Pseudomonas Acidovorans DMR-11 isolated from peat biofilter. Biotech. Lett. 3:223-228 (1991).
(27) Visscher, PT, Quist, P. and van Gemerden H. Methylated sulfur compounds in microbial mats: In situ concentrations and metabolism by a colorless sulfur bacterium. Appl. Environ. Microbiol. 57:1758-1763 (1991).

(28) Kiene, RP and Bates, TS. Biological removal of dimethyl sulphide from sea water. Nature 345:702-705 (1990).
(29) Smith NA. and Kelly DP. isolation and physiological characterization of autotrophic sulphur bacteria oxidizing dimethyl disulphide as sole source of energy. J. Gen. Microbiol. 134:1407-1417 (1988).
(29a) Kuene, JG. and Beudeker, RF. Microbiology of thiobacilli and other sulphur-oxidizing autotrophs, mixotrophs and heterotrophs. Phil. Trans. R. Soc. Lond. B298:473-497 (1982).
(30) Smet, E., Verstraete, W. and Van langenhove, H. Enrichment culture for the oxidation of organic sulphur compounds. Med. Fac. landbouww. Univ. Gent. 57/4a:1725-1728 (1992).
(31) Kelly DP. and Smith NA. Organic sulfur compounds in the environment. Biogeochemistry, microbiology, and ecological aspects. Adv. Microbiol. Ecol. 11:345-385 (1989).
(32) Kodota, H. and Ishida, Y. Production of volatile sulfur compounds by microorganisms. Ann. Rev. Microbiol. 26:127-138 91972).
(33) Bremner, JM. and Steele, CG. Role of microorganisms in the atmospheric sulfur cycle. Adv. Microbiol Ecol. 2:155-201 (1978).
(34) Wagner, C., Lusty, SM., Kung, H-F., and Rogers, NL. Preparation and properties of trimethylsulfonium-tetrahydrofolate methyltransferase. J. Biol. Chem. 242:1287-1293 (1967).
(35) Wagner, C., Lusty, SM., Kung, H-F., and Rogers, NL. Trimethylsulfonium-tetrahydrofolate methyltransferase, a novel enzyme in the utilization of 1 carbon units. J. Biol. Chem. 241:1923-1924 (1966).
(36) Kung, H-F. and Wagner, C. Oxidation of C_1 compounds by Pseudomonas Sp. MS. Biochem. J. 116:357-365 (1970).
(37) Hampton, D. and Zatman LJ. The metabolism of tetramethylammonium chloride by bacterium 5H2. Biochem. Soc. Trans. 1:667-668 (1973).
(38) Mizoguchi, T., Sato, T. and Okabe, T. New sulfur-oxidizing bacteria capable of growing heterotrophically, Thiobacillus rubellus nov. sp. and Thiobacillus delicatus nov. sp. J. Ferment. Technol. 54:181-191 (1976).
(39) Harrison, AP. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. Ann. Rev. Microbiol. 38:265-292 (1984).
(40) Huber, H. and Stetter, KO. Thiobacillus cuprinus sp. nov., a novel facultatively organotrophic metal-mobilizing bacterium. Appl. Environ. Microbiol. 56:315-322 (1990).
(41) Zavarzin, GA. A heterotrophic satellite of Thiobacillus ferrooxidans. Microbiol. 41:323-324 (1972).
(42) Wichlacz, PL. and Unz, Rp. Acidophilic, heterotrophic bacteria of acidic mine waters. Appl. Environ. Microbiol. 41:1254-1261 (1981).
(43) Wichlacz, PL. Unz, RF. and Langworthy, TA. Acidiphilium angustum sp. nov., Acidiphilium facilis sp. nov., and Acidiphilium rubrum sp. nov.: Acidophilic heterotrophic bacteria isolated from acidic coal mine drainage. Int. J. System. Bact. 36:197-201 (1986).

(44) Lobos, JH., Chisolm, TE., Bopp, LH. and Holmes, DS. Acidiphilium organovorum sp. nov., an acidophilic heterotroph isolated from a Thiobacillus ferrooxidans culture. Int. J. System. Bact. 36:139-144 (1986).
(45) Kishimoto, N. and Tano, T. Acidophilic heterotrophic bacteria isolated from acidic mine drainage, sewage, and soils. J. Gen. Appl. Microbiol. 33:11-25 (1987).
(46) Bhattacharyya, S., Chakrabarty, BK. Das, A. Kundu, PN. and Banerjee, PC. Acidiphilium symbioticum sp. nov., an acidophilic heterotropic bacterium, from Thiobacillus ferrooxidans cultures isolated from Indian mines. Can. J. Microbiol. 37:78-85 (1990).
(46a) Roberto, FF., Glenn, AW., Bulmer, D., Bruhn, DF. and Ward, TE. Genetic manipulation of acidophilic bacteria which are potentially applicable in coal beneficiation. Amer. Chem. Soc., Div. Fuel Chem. 35:868-874 (1990).
(47) Matin, A. Keeping a neutral cytoplasm; the bioenergetics of obligate acidophiles. FEMS Microbiol. Rev. 75:307-318 (1990).
(48) Norris, PR. and Ingledew, WJ. Acidophilic Bacteria: Adaptations and Applications. In Molecular Biology and Biotechnology of Extremophiles. Ed. RA. Herbert and RT. Sharp. Blackie Publishing Group, U.K. 1992.
(49) Bakker, EP. The role of alkali-cation transport in energy coupling of neutrophilic and acidophilic bacteria: an assessment of methods and concepts. FEMS Microbiol. Rev. 75:319-334 (1990).
(50) Booth, IR. Regulation of cytoplasmic pH in bacteria. 49:359-378 (1985).
(51) Guay, R. and Silver, M. Thiobacillus acidophilus sp. nov.; isolation and some physiological characteristics. Can. J. Microbiol. 21:281-288 (1975).
(52) Salsbury, RL. and Merricks, DL. Production of methanethiol and dimethyl sulfide by rumen micro-organisms. Plant Soil 43:191-209 (1975).
(53) Kargi, F. Microbial methods for desulfurization of coal. Trends Biotech. 4:293-297 (1986).
(54) Kilbane, JJ. Desulfurization of coal: the microbial solution. Trend Biotech. 7:97-101 (1989).
(55) Kilbane JJ. Microbial Removal of Organic Sulfur from Coal: Current Status and research Needs. In Bioprocessing and Biotreatment of Coal. Ed. DL. Wise, Marcel Dekker, N.Y. Chap. 27, 1990.
(56) ElSawy, A. and Gray, D. A critical review of biodesulphurization systems for removal of organic sulphur from coal. Fuel 70:591-594 (1991).
(57) Monticello, DJ. and Finnerty, WR. Microbial desulfurization of fossil fuels. Ann. Rev. Microbiol. 39:371-389 (1985).
(58) Foght, JM., Fedorak, PM. and Westlake, WS. Microbial desulfurization of crude oil. Proc. Fourth Int. Conf. Heavy Crude and Tar Sands. 5:365-373 (1988).
(59) Malik, KA. Microbial removal of organic sulphur from crude oil and the environment: some new perspectives. Process Biochem. 13:10-35 (1978).

(60) Isbister,JD. and Kobylinski, EA. Microbial Desulfurization of Coal. In Processing and Utilization of High Sulfur Coals, Vol 9 in Coal Science and Technology Series. Ed. YA. Attia, Elsevier, Amsterdam, p 627, 1985.
(61) Monticello, DJ., Bakker, D. and Finnerty WR. Plasmid-mediated degradation of dibenzothiophene by Pseudomonas species. Appl. Environ. Microbiol. 49:756-760 (1985).
(62) Hou, CT. and Laskin, AI. Microbial conversion of dibenzothiophene. Dev. Ind. Microbiol. 17:351-362 (1976).
(63) Rai, C. and Reyniers, JP. Microbial desulfurization of coals by organisms of the genus Pseudomonas. Biotech. Prog. 4:225-230 (1988).
(64) Mormile, MR. and Atlas, RM. Mineralization of the dibenzothiophene biodegradation products 3-hydroxy-2-formyl benzothiophene and dibenzothiophene sulfone. Appl. Environ. Microbiol. 54:3183-3184 (1988).
(65) Yamada, K., Minoda, Y, Kodama, K., Nakatani, S. and Akasaki, T. Microbial conversion of petro-sulfur compounds. Part 1. Isolation and identification of dibenzothiophene-utilizing bacteria. Agric. Biol. Chem. 32:840-845 (1968).
(66) Laborde, AL. and Gibson, DT. Metabolism of dibenzothiophene by a Beijerinckia Species. Appl. Environ. Microbiol. 34:783-790 (1977).
(67) Kilbane, JJ. and Bielaga, BA. Microbial removal of organic sulfur from coal: A molecular genetics approach. Gas, Oil, Coal, Environ. Biotech. 2:317-30 (1990).
(68) Kilbane, JJ and Jackowski, K. Biodesulfurization of water-soluble coal-derived material by Rhodococcus rhodochrous IGTS8. Biotech. Bioeng. 40:1107-1114 (1992).
(69) Omori, T., Monna, L., Saiki, Y. and Kodoma, T. Desulfurization of dibenzothiophene by Corynebacterium sp. strain SY1. Appl. Environ. Microbiol. 58:911-915 (1992).
(70) Stoner, DL., Wey, JE., Barrett, KB. Jolley, JG. Wright, RB. and Dugan, JG. Modification of water-soluble coal-derived products by dibenzothiophene-degrading microorganisms. Appl. Environ. Microbiol. 56:2667-2676 (1990).
(71) Isbister, JD., Wyza, RE. and Lippold, J. Bioprocessing of Coal. In Environmental Biotechnology: Reducing Risks from Environmental Chemicals through Biotechnology. Ed. GS. Omenn, Plenum Press, N.Y. 1988, p280-293.
(72) Mattoon, JR., Schwartz, D. Mooney, M. Neus, P. and Campbell, A. Isolation of microorganisms which can extract organic sulfur from solubilized coal. Gas, Oil, Coal, Environ. Biotech. 2:305-315 (1990).
(73) van Afferden, M., Schacht, S., Klein, J. and TrÜper, HG. Degradation of dibenzothiophene by Brevibacterium sp. DO. Arch. Microbiol. 153:324-328 (1990).
(74) Kargi, F. and Robinson, JM. Microbial oxidation of dibenzothiophene by the thermophilic organism Sulfolobus acidocaldarius. Biotech. Bioeng. 24:687-690 (1984).
(75) Kitchell, JP., Nochur, SV., Marquis, JK., Bazylinski, DA. and Jannasch, H. Microbiol oxidation of sulfur in dibenzothiophene. Resources Conser. Recycl. 5:255-263 (1991).

(76) Kodoma, K., Umehara, K., Shimizu, K., Nakatani, S., Minoda, Y. and Yamada, K. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agr. Biol. Chem. 37:45-50 (1973).
(77) Ochman, O., Klubek, B. Boydstun, J., Clark, D. and Nabe, S. Mineralization of S from dibenzothiophene, dibenzothiophene sulphone and benzene sulphonic acid by soil isolates. Microbios 63:79-91 (1990).
(78) Gallagher, JR., Olson, ES. and Stanley, DC. Microbial desulfurization of dibenzothiophene: A sulfur-specific pathway.
FEMS Microbiol. Lett. 107:31-36 (1993).
(79) Roberto, FF., Glenn, AW., Bulmer, D. and Ward, TE. Genetic transfer in acidophilic bacteria which are potentially applicable in coal beneficiation. Fuel 70:595-598 (1991).
(80) Glenn, AW>, Roberto, FF. and Ward, TE. Transformation of Acidiphilium by electroporation and conjugation. Can. J. Microbiol. 38:387-393 (1992).
(81) Sagardia, F., Rigau, JJ., Martinez-Lahoz, A., Fuentes, F., Lopez, C. and Flores, W. Degradation of benzothiophene and related compounds by a soil Pseudomonas in an oil-aqueous environment. Appl. Microbiol. 29:722-725 (1975).
(82) Foght, JM. and Westlake DWS. Degradation of polycyclic hydrocarbons and aromatic heterocycles by a Pseudomonas species. Can. J. Microbiol. 34:1135-1141 (1988).
(83) Foght, FM, Fedorak, PM, Gray, MR. and Westlake, DWS. Microbial Desulfurization of Petroleum. In Microbiol Mineral Recovery, Ed. HL. Ehrlich and CL. Brierley, McGraw-Hill Publishing Co., N.Y. 1990, 379-407.
(84) Fedorak, PM., Payzant, JD., Montgomery, DS. and Westlake, DWS. Microbial degradation of n-alkyl tetrahydrothiophenes found in petroleum. Appl. Environ. Microbial. 54:1243-1248 (1988).
(85) KÖehler, M., Genz, I-L., Schicht, B. and Eckart, V. Microbial desulfurization of petroleum and heavy petroleum fractions. 4. Anaerobic degradation of organic sulfur compounds of petroleum. Zbl. Mikrobiol. 139:239-247 (1984)
(86) Kim, HY., Kim, TS. and Kim, BH. Degradation of organic sulfur compounds and the reduction of dibenzothiophene to biphenyl and hydrogen sulfide by Desulfovibrio desulfuricans M6. Biotech. Lett. 12:761-764 (1990).
(87) Eckart, V., KÖhler, M. and Hieke, W., Microbial desulfurization of petroleum and heavy petroleum fractions. 5. Anaerobic desulfurization of Romashkino petroleum. Zbl. Mikrobiol. 141:291-300 (1986).
(88) Fedorak, PM. and Westlake, DWS. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. 29:291-296 (1983).
(89) Setti, L., Rossi, M., Lanzarini, G., and Pifferi, PG. The effect of n-alkanes in the degradation of dibenzothiophene and of organic sulfur compounds in heavy oil by a Pseudomonas sp. Biotech Lett. 14:515-520 (1992).

(90) Bacterial membrane extracts and enzymes of Rhodococcus rhodochrous and Bacillus sphaericus; application in organic carbon-sulfur bond cleavage and fossil fuel e.g. oil, coal desulfurization. Inst. Gas-Technology Patent EP 445896, 1991.
(91) Preparation of stable single-phase solution of water-in-oil emulsion -oil desulfurization using microorganism, animal or plant cell or derivative and surfactant. Eniricerche S.p.A., Patent EP 409314, 1991.
(92) Monticello, DJ. Bakker, D. and Finnerty, WR. Plasmid-mediated degradation of dibenzothiophene by Pseudomonas species. Appl. Environ. Microbiol. 49:756-760 (1985).
(93) Odu, CTI. Fermentation characteristics and biochemical reactions of some organisms isolated from oil-polluted soils. Environ. Pollut. 15:271-276 (1978).
(94) Sorkhoh, NA., Ghannoum, MA., Ibrahim, AS. Stretton, RJ. and Radwan, SS. Crude oil and hydrocarbon-degrading strains of Rhodococcus rhodochrous isolated from soil and marine environments in Kuwait. Environ. Pollut. 65:1-17 (1990).
(95) Deshpande, MS., Rale, VB. and Lynch JM. Aureobasidium pullulans is applied microbiology: A status report. Enzyme Microb. Technol. 14:514-527 (1992).
(96) Cohen, MS. and Gabriele PD. Degradation of coal by the fungi Ployporus versicolor and Poria monticola. Appl. Environ. Microbiol. 44:23-27 (1982).
(97) Scoot, CD., Strandberg, GW. and Lewis, SN. Microbial solubilization of coal. Biotech. Prog. 2:131-139 (1986).
(98) Faison, BD. Biological coal conversions. Crit. Rev. Biotech. 11:347-366 (1991).
(99) Faison, BD. and Lewis, SN. Production of coal-solubilizing activity by Paecilomyces sp. During submerged growth in defined liquid media. Appl. Biochem. Biotech. 20/21:743-752 (1989).
(100) Reiss, J. Studies on the solubilization of German coal by fungi. Appl. Microbiol. Biotech. 37:830-832 91992).
(101) Ward, B. Lignite-degrading fungi isolated from a weathered outcrop. System. Appl. Microbiol. 6:236-238 (1985).
(102) Holland, HL., and Carter, IM. The mechanism of sulphide oxidation by Mortierella isabellina NRRL 1757. Can. J. Chem. 60:2420-2425 (1982).
(103) Holland, HL., Khan, SH., Richards, D. and Riemland, E. Biotransformation of polycyclic aromatic compounds by fungi. Xenobiotica, 16:733-741 (1986).
(104) Crawford, DL. and Gupta, RK. Oxidation of dibenzothiophene by Cunninghamella elegans. Curr. Microbiol. 21:229-231 (1990).
(105) Phae, C-G. and Shoda, M. A new fungus which degrades hydrogen sulfide, methanethiol, dimethyl sulfide and dimethyl disulfide. Biotech. Lett. 13:375-380 (1991).
(106) Schreiner, RP., Stevens, SE. and Tien, M. Oxidation of thianthrene by the ligninase of Phanerochaete chrysosporium. Appl. Environ. Microbiol. 54:1858-1860 (1988).
(107) Faison, BD. Microbial conversions of low rank coals. Biotech. 9:951-956 91991).

(108) Paison, BD., Clark, TM., Lewis, SN., Ma, CY., Sharkey, DM. and Woodward, CA. Degradation of organic sulfur compounds by a coal-solubilising fungus. Appl. Biochem. Biotech. 28/29:237-251 (1991).
(109) Langworthy, TA. Microbial Life in Extreme pH Values. in Microbial Life in Extreme Environments. Ed. DJ. Kushner, Academic press, NY. 1978, 279-315.
(110) Gould, WD., Fujikawa, JI. and Cook, FD. A soil fungus tolerant to extreme acidity and high salt concentrations. Can. J. Microbiol. 20:1023-1027 (1974).
(111) Raj, HG., Saxena, M. and Allameh, A. Metabolism of foreign compounds by fungi. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 881-904.
(112) Nandan, R. and Raisuddin S. Fungal Degradation of Industrial Wastes and Wastewater. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 931-961.
(113) Field, JA., de Jong, E., Feijoo-Costa, G. and de Bont JAM. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotech. 11:44-49 (1993).
(114) Lindley, ND. Hydrocarbon-degrading Yeasts and Filamentous Fungi of Biotechnological Importance. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 905-929.
(115) Elander, RP.and Lowe, DA. Fungal Biotechnology: An Overview. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 1-34.
(116) Lowe, DA. Fungal Enzymes. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 681-706.
(117) Weete, JD and Gandhi, S. Potential for Fungal Lipids in Biotechnology. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 377-400.
(118) Bumpus, JA., Tien, M., Wright, D. and Aust, SD. Oxidation of persistent environmental pollutants by a white rot fungus. Science, 228:1434-1436 (1985).
(119) Kushner, DJ. Molecular adaptations of enzymes, metabolic systems and transport systems in halophilic bacteria. FEMS Microbiol. Rev. 39:121-127 (1986).
(120) Zaccai, G. and Eisenberg, H. Halophilic proteins and the influence of solvent on protein stabilization. Trends Biochem. Sci. 15:333-337 (1990).
(121) Rodriguez-Valera, F. Biotechnological potential of halobacteria. Biochem. Soc. Symp. 58:135-147 (1991).
(122) Keradjopoulos, D. and Holldorf, AW. FEMS Microbiol. Lett. 1:179-182 (1977).
(123) Oren, A. A thermophilic amyloglucosidase from Halobacterium sodomense, a halophilic bacterium from the Dead Sea. Curr. Microbiol. 8:225-230 (1983).

(124) Owusu, RK. and Cowan, DA. Correlation between microbial protein thermostability and resistance to denaturation in aqueous:organic solvent two-phase systems. Enzyme Microb. Technol. 11:568-574 (1989).
(125) Hough, DW. and Danson MJ. Archaebacteria: ancient organisms with commercial potential. Let. Appl. Microbiol. 9:33-39 (1989).
(126) Cowan, DA. Enzymes from thermophilic archaebacteria: current and future applications in biotechnology. Biochem. Soc. Symp. 58:149-169 (1991).
(127) Satyanarayana, T., Johri, BN., and Klein, J. Biotechnological Potential of Thermophilic Fungi. In Handbook of Applied Mycology, Vol. 4. Fungal Biotechnology, Ed. DK. Arora, RP. Elander, and KG. Mukerji. Marcel Dekker, NY, 1992, 729-761.
(128) Deetz, JS., and Rozzell, JD. Enzyme-catalyzed reactions in non-aqueous media. Trends Biotech. 6:15-19 (1988).
(129) Klibanov, AM. Enzymatic catalysis in anhydrous organic solvents. Trend Biochem. Sci. 14:141-144 (1989).
(130) Kargi, F. Biological oxidation of thianthrene, thioxanthene and dibenzothiophene by the thermophilic organism Sulfolobus acidocaldarius. Biotech. Lett. 9:478-482 (1987).
(131) Constanti, M., Giralt, J., Bordons, A. and Norris, PR. Interactions of thiophenes and acidophilic, thermophilic bacteria. Appl. Biochem. Biotech. 34/35:767-776 (1992).
(132) Norris, PR. Thermoacidophilic archaebacteria: potential applications. Biochem. Soc. Symp. 58:171-180 (1991).
(133) Sharma, HSH. Economic importance of thermophilous fungi. Appl. Microbiol. Biotechnol. 31:1-10 (1989).